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Extent of DNA 2-hydroxyethylation by *N*-nitrosomethylethylamine and *N*-nitrosodiethylamine *in vivo*

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At low doses, *N*-nitrosomethylethylamine (NMEA) selectively produces liver tumors in rats, whereas β-trideuterated NMEA also includes esophageal carcinomas under these conditions. Since deuteration is capable of retarding enzymic hydroxylation, these studies suggest that β-hydroxylation plays a significant role in the organ specificity of NMEA. To test the hypothesis that this metabolic pathway occurs *in vivo* to yield a hydroxyethylating intermediate, we have determined the extent of hydroxyethylation of hepatic DNA in male Fischer 344 rats following a single i.p. injection of [1-ethyl-^{14}C]NMEA (6.3 mg/kg, 4 h survival). After hydrolysis in 0.1 M HCl, DNA purines were analysed by cation exchange chromatography. Of the major alkylpurines identified, 7-ethylguanine (7-etG) (6.7 μmol/mol guanine) and O^6-ethylguanine (4.1 μmol/mol guanine) comprised 13 and 8% of the eluted radioactivity, respectively. 7-(2-Hydroxyethyl)guanine (7-heG) was the only hydroxyethyl adduct detectable, and comprised less than 2% of the amount of 7-etG. 3-Ethylguanine and 3- and 7-ethyladenine were also identified as products of NMEA metabolism. Similar analyses were carried out on hepatic DNA from rats treated with *N*-nitrosodi[1-^{14}C]ethylamine (6.9 mg/kg, 4 h survival). Only trace amounts of 7-heG could be detected. The very low concentrations of β-hydroxyethylated DNA bases observed suggest that this route of metabolism does not contribute significantly to the carcinogenicity of these compounds.

Introduction

The study of enzymic hydroxylation of aliphatic *N*-nitroso compounds at other than the α-carbon atom has previously focused on long-chain dialkylnitrosamines (1,2,3). There is evidence that *N*-nitroso compounds containing an ethyl group are also ω-hydroxylated (4). The involvement of β-hydroxylation as a modifying factor in the carcinogenicity of *N*-nitrosomethylethylamine (NMEA*) has been suggested by the altered tissue specificity of NMEA deuterated on the β-carbon of the ethyl group (5,6). This deuterated analog produces significantly more esophageal tumors than does the parent compound, probably due to impaired β-hydroxylation (7,8). We have previously shown that NMEA is hydroxylated at both the methyl group and at the α-position of the ethyl group to yield an ethylating and a methylating species *in vivo*, respectively (9). The purpose of the present study was to determine to what extent β-hydroxylation of NMEA (Figure 1) yields a hydroxyethylating intermediate. White *et al.* (10) have recently shown that porphyrins in the livers of mice receiving *N*-nitrosodi[1-^{14}C]ethylnitrosamine ([1-ethyl-^{14}C]NDEA) were hydroxyethylated rather than ethylated. We have, therefore, also studied DNA hydroxyethylation by [1-ethyl-^{14}C]NDEA. The pattern of alkylated DNA bases was analysed on a cation exchange chromatography system in which 7-(2-hydroxyethyl)guanine (7-heG) was clearly separated from other DNA purines. The results provide evidence that NMEA and *N*-nitrosodiethylamine (NDEA) are β-hydroxylated *in vivo* and subsequently hydroxyethylate DNA, although to a very small extent.

*Abbreviations: NMEA, *N*-nitrosomethylethylamine; NDEA, *N*-nitrosodiethylamine: 7-etG, 7-ethylguanine; 7-heG, 7-(2-hydroxyethyl)guanine; O^6-etG, O^6-ethylguanine; O^6-heG, O^6-(2-hydroxyethyl)guanine; 3-etG, 3-ethylguanine; 3-etA, 3-ethyladenine; 7-etA, 7-ethyladenine; G, guanine; A, adenine.

Materials and methods

Chemicals

NMEA ^{14}C-labeled in the 1-ethyl position was prepared as previously described (9). [1-Ethyl-^{14}C]NDEA was obtained from New England Nuclear (Boston, MA). Both nitrosamines were dissolved in physiological saline at specific activities of 17 mCi/mmol for [1-ethyl-^{14}C]NDEA and 14 or 32 mCi/mml for [1-ethyl-^{14}C]-NMEA. The concentrations of solutions administered were 2.8 mM for [1-ethyl-^{14}C]NDEA and 12 mM for [1-ethyl-^{14}C]NMEA at 14 mCi/mmol and 1.6 mM at 32 mCi/mmol. The standards 7-heG and O^6-(2-hydroxyethyl)guanine (O^6-heG) were a gift from Dr D.Ludlum, 7-ethylguanine (7-etG) was from Dr W.Lijinsky, and O^6-ethylguanine (O^6-etG) was purchased from EMKA-Chemie (Markgröningen, FRG).

Animals and treatments

Male Fischer 344 rats weighing 100–150 g were obtained from Charles River Wiga (FRG) and given food and water *ad libitum*. The two nitrosamines were administered i.p. in physiological saline. In the first experiment, [1-ethyl-^{14}C]-NMEA was administered to six animals at 0.05 mmol/kg (sp. act. 14 mCi/mmol). In a second experiment, the same compound was given to one animal at a dose of 6.3 mg/kg (0.07 mmol/kg, sp. act. 32 mCi/mmol); the survival time was 4–5 h. [1-Ethyl-^{14}C]NDEA was given to two animals at a dose of 6.9 mg/kg (0.07 mmol/kg) with a survival time of 4 h.

Analysis

DNA was isolated by phenol extraction and adsorption onto hydroxylapatite as

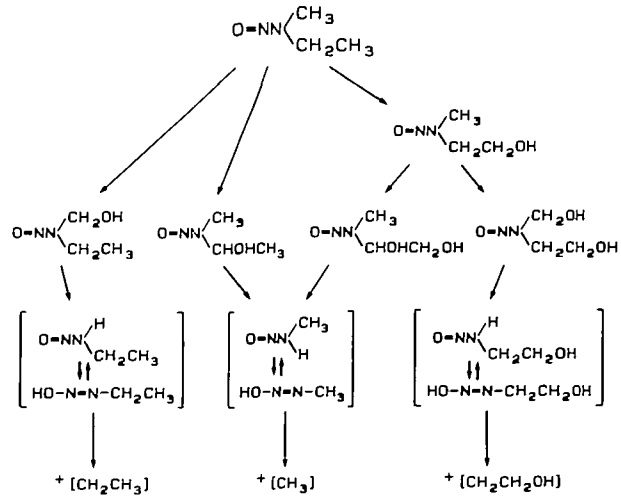

Fig. 1. Proposed pathways for the metabolism of *N*-nitrosomethylethylamine *in vivo*, including β-hydroxylation.

previously described (9). It was then depurinated for 20 h at 37°C in 0.1 M HCl. When authentic 7-heG and O^6-heG were submitted to this treatment, no degradation of the standards could be observed by u.v. detection following h.p.l.c. on a 0.45 × 25 cm Partisil SCX column eluted with 50 mM $NH_4H_2PO_4$ and 10% (v/v) methanol (pH 2.0). In the first experiment, the DNA purines from 16 g of pooled livers from animals treated with 0.05 mmol/kg [1-ethyl-^{14}C]NMEA was loaded onto a Sephasorb-HP column (1 × 40 cm) and eluted with water at a flow rate of 1.5 ml/min (fraction volume, 3.5 ml). Under these conditions, 7-heG was previously found to elute at fraction 14. Fractions 12–16 were pooled, evaporated under a stream of purified air to 200 μl and analysed by h.p.l.c. as described above, after the addition of cold 7-heG marker. Fractions were collected every 0.3 min and counted for radioactivity after the addition of 5 ml Riatron scintillation cocktail. The chromatographic system used to separate radiolabeled purine adducts in the second experiment using [1-ethyl-^{14}C]NMEA at a higher specific activity and using [1-ethyl-^{14}C]NDEA was that described by Lawley *et al.* (11). Authentic 7- and O^6-heG as well as 7-etG and O^6-etG were added to the hydrolysates as optical markers. The resulting mixture was brought to 0.3 M ammonium formate (pH 8.9) by the addition of 3 M ammonium formate and 1 M sodium hydroxide and loaded onto a 70 × 1 cm column of Bio-Rad AG 50W-X4 minus 400 mesh resin (ammonium form). DNA bases were eluted with 0.3 M ammonium formate (pH 8.9) at a flow rate of 7 ml/h. This cation exchange system has the advantage that 7-heG, the major hydroxyethylated adduct expected, elutes far from the solvent front and away from other bases. Peaks were identified by their retention times relative to adenine and added optical markers. Radioactivity was quantified in 3.5 ml fractions following the addition of 6 ml Riatron.

Results

In the first experiment using [1-ethyl-^{14}C]NMEA at 14 mCi/mmol, a small peak of radioactivity was observed to co-chromatograph with 7-heG after analysis by h.p.l.c. (data not shown). Since the sample was analysed by two separate chromatographic systems with a concentration step in between, it was not possible to reliably correct for recovery and thus the amount of adduct could not be quantified in terms of μmol 7-heG/mol guanine.

The amounts of ethylated and hydroxyethylated DNA purines produced by NMEA and NDEA detected following separation on AG 50-X4 resin are shown in Table I. In all analyses, 3-ethylguanine (3-etG), 3-ethyladenine (3-etA), 7-etG, 7-ethyladenine (7-etA) and O^6-etG were identified. In addition, DNA from animals treated with [1-ethyl-^{14}C]NMEA showed a small radiolabeled peak which co-chromatographed with 7-heG (Figure 2). This peak contained 1.3% of the radioactivity found in the 7-etG peak. In the DNA of animals treated with [1-ethyl-^{14}C]NDEA, a radioactive peak with the retention time of 7-heG also was present, but its height was less than twice the background activity (Figure 3). From the specific activity of [1-ethyl-^{14}C]NDEA administered it was calculated that the amount of 7-heG present was below 0.4 μmol/mol guanine, i.e. less than 1% of the 7-etG in the same chromatogram. O^6-heG was not detectable, but this may have been due to partial co-elution with 7-etA. The relative amounts of the remaining adducts produced by [1-ethyl-^{14}C]-NMEA were comparable with those produced by [1-ethyl-^{14}C]-NDEA, with the one notable exception that the O^6-etG to 7-etG ratio was higher for [1-ethyl-^{14}C]NMEA than for [1-ethyl-^{14}C]NDEA.

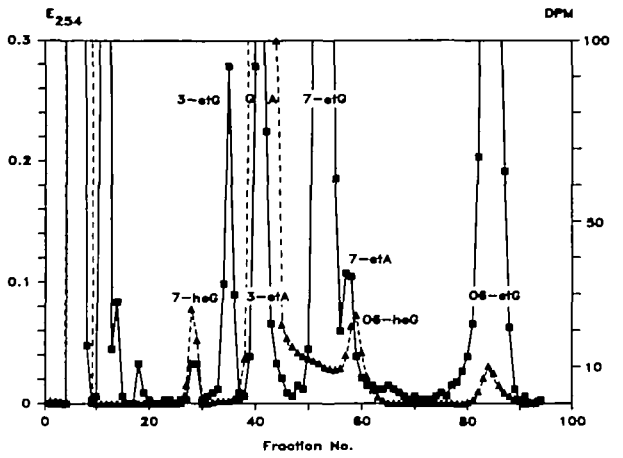

Fig. 2. Ion exchange chromatographic profile of hepatic DNA purines from a male Fischer 344 rat injected i.p. with 6.3 mg/kg [1-ethyl-^{14}C]NMEA (32 mCi/mmol): --▲--▲--, u.v. (254 nm) absorbance; —■—■—, radioactivity (d.p.m.).

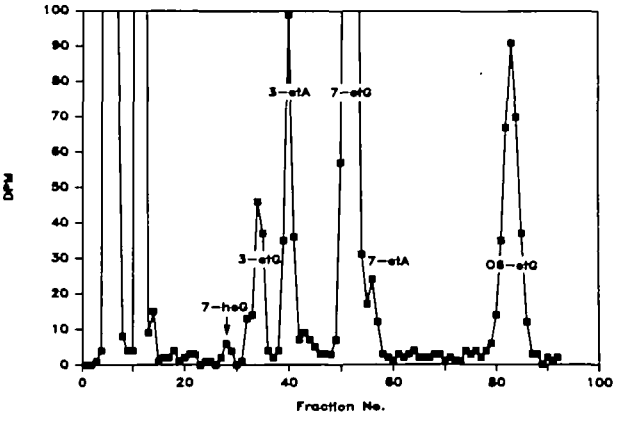

Fig. 3. Chromatographic profile of hepatic DNA purines from male Fischer 344 rats injected i.p. with 6.9 mg/kg [1-ethyl-^{14}C]NDEA (17 mCi/mmol): —■—■—, radioactivity (d.p.m.).

Table I. DNA adducts produced by ethylating nitroso compounds *in vivo*

Adduct	Retention time relative to adenine (=100)	[1-Ethyl-^{14}C]NMEA[a] (6 3 mg/kg)		[1-Ethyl-^{14}C]NDEA[b] (6.9 mg/kg)	
		Percentage of total d.p.m. eluted	μmol/mol G or A	Percentage of total d.p.m. eluted	μmol/mol G or A
Front	15	73		66	
7-heG	69	0.17	0.09	<0.2	<0.4
3-etG	85	1.3	0.66	2.0	3.6
3-etA	99	3.2	1.2	3.5	4.6
7-etG	129	13	6.7	19	34
7-etA	139	0.75	0.27	0.97	1.3
O^6-etG	204	8.0	4.1	6.9	13
O^6-etG/7-etG		0.61		0.36	
7-heG/7-etG		0.013		<0.01	

[a]Single analysis of DNA from the liver of one rat.
[b]Mean of two determinations of DNA from pooled livers of two rats.

Discussion

The present investigation has shown that β-hydroxylation of NMEA occurs *in vivo*, yielding a hydroxyethylating intermediate. Following administration of 0.07 mmol/kg of [1-ethyl-^{14}C]-NMEA, a radioactive peak co-eluting with 7-heG was clearly identifiable and amounted to 1.3% of the 7-etG present. In the case of [1-ethyl-^{14}C]NDEA, the extent of β-hydroxyethylation at the 7-position of guanine was less than 1% of the extent of ethylation. From *in vitro* studies on alkylation by *N*-nitroso-hydroxyethylurea one would expect an O^6/7-heG ratio close to 0.72 (12). In the present study we could not detect O^6-heG, probably due to the low extent of its formation and partial co-elution with 7-etA (Figure 2). In addition, the possibility exists that this promutagenic base was partially repaired during the course of the experiment. O^6-heG is a substrate for O^6-alkylguanine–DNA alkyltransferase, although the rate of repair is considerably slower than for O^6-methylguanine or O^6-etG (12, 13). The relative amounts of ethylated bases observed (Table I) correspond closely to those determined in DNA treated with *N*-nitrosoethylurea *in vitro* (14). It is noteworthy that the O^6-/7-etG ratio is markedly lower in the DNA from animals receiving [1-ethyl-^{14}C]NDEA than from animals receiving [1-ethyl-^{14}C]NMEA (Table I); most likely this reflects partial saturation of O^6-alkylguanine–DNA alkyltransferase by O^6-methylguanine, levels of which exceed those of O^6-etG by approximately 30-fold following administration of [1-ethyl-^{14}C]NMEA (9).

From the work of White *et al.* (10) we expected that β-hydroxylation of ethylating nitrosamines might constitute a major metabolic pathway. Following the administration of [1-ethyl-^{14}C]-NDEA (1 mmol/kg) they found in mouse liver hydroxyethyl-protoporphyrin IX rather than an ethylprotoporphyrin. They suggested that this was due to β-hydroxylation of NDEA followed by α-C hydroxylation to yield a hydroxyethyldiazonium ion as the ultimate reactant, analogous to the bioactivation of NMEA shown in Figure 1. When taking DNA modification rather than reaction with proteins as a biological endpoint of NDEA metabolism in rats, it would seem that β-hydroxylation is either a minor pathway or that subsequent activation of β-hydroxylated NMEA or NDEA leading to DNA hydroxyethylation is not a favoured reaction. In the latter case, β-hydroxylation of NMEA could serve as an effective detoxification pathway. We have not, however, ruled out the possibility that β-hydroxylated NMEA may undergo subsequent α-hydroxylation of the hydroxyethyl moiety to yield a methylating agent (Figure 1). The contrasting type of alkylation of prophyrins and deoxyribonucleotides may reflect an intracellular heterogeneity of nitrosamine metabolism and/or differences in the respective nucleophiles participating in the alkylation reaction as well as species differences.

Our observations are in agreement with numerous studies on DNA alkylation by NDEA (15,16), NMEA (9) and *N*-nitroso-ethylurea (17). All these *in vivo* studies clearly demonstrate that DNA ethylation is the major type of adduct formed by transfer of the 2-carbon *N*-substituent to DNA. In addition, other workers have very recently obtained evidence for DNA 2-hydroxyethylation in independent investigations with NDEA (H.G.Floss and Y.-H.Heur, personal communication).

Carcinogenicity studies with *N*-nitrosohydroxyethylurea indicate that DNA hydroxyethylation *in vivo* is an initiating event at least as potent as DNA ethylation (18). However, the very low extent of DNA hydroxyethylation observed in the present study suggests that enzymic β-hydroxylation of ethylating nitroso compounds does not contribute significantly to their carcinogenicity. The shift in organ specificity by β-trideuteration of NMEA from liver to esophagus is, therefore, unlikely to result from different levels of DNA hydroxyethylation. The possibility exists that β-trideuteration of NMEA retards its overall metabolism in rat liver, allowing increased exposure of extra-hepatic tissues.

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